



Docket No. 55591 RCE (71699)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.131

The undersigned declare as follows:

1. We are co-inventors of the above-identified patent application assigned to The Johns Hopkins University.
2. Prior to September 1998, we conceived of and then diligently reduced to practice the methods of introducing into endothelial cells of an autologous vein grafts from a mammal an effective amount of at least one nucleic acid encoding thrombomodulin (TM), NF- κ B inhibitor, or a functional fragment of TM; provided that when the agent is thrombomodulin, the nucleic acid further encodes the NF- κ B inhibitor, wherein the introducing is performed ex vivo or by direct injection into the graft, and transplanting the vein graft into the mammal as disclosed and claimed in the above-identified patent application.
3. We diligently worked to reduce the methods to practice until the filing of the provisional application on May 22, 2000.
5. Attached as Exhibit 1, Figures 1 – 20, are true and accurate copies of laboratory notebook records with dates deleted. The notebook records demonstrate the conception, reduction to practice and diligence from conception to the filing of the application. The exhibits show that the constructs for the expression were received prior to September 1998 representing proof of the conception of the methods of using thrombomodulin to prevent early vein graft thrombosis as described in paragraph 2 above (Figure 1).

6. Figure 2 shows successful adenovirus-mediated gene transfer of and expression of B-galactosidase marker genes in rabbit vein grafts. Figure 3 demonstrates that we were also transducing rabbit vein graft with the adenovirus vector expressing human TM (AdTMh5; Figure 3). Figure 4 demonstrates that we were able to quantify native TM expression in vein grafts by harvesting rabbit vein grafts at various time points. Figure 5 is a page from Dr. Antony Kim's notebook demonstrating TM expression over time (TMTC series of rabbits) outlining a visual scoring system devised to quantify native TM expression in rabbit vein grafts.

7. Figure 6, a page from Dr. Rade's notebook, depicts the continued generation of rabbits for TM quantification (C6W) and the transduction of rabbit vein grafts with control adenovirus (Adl312). Figure 7, a page from Dr. Kim's notebook, details a digital scoring system for TM expression in vein grafts. Figure 8, a log from Dr. Rade's notebook demonstrates transduction of a series of rabbit vein grafts with a different control adenovirus (AdRNull-1). Figures 9 and 10, from Dr. Kim's notebook, document TM expression in rabbit vein grafts using the digital imaging system.

8. Figure 11, a page from Dr. Rade's notebook, shows a series of rabbit vein grafts transduced with AdTMh5. Figures 12-14, from Dr. Kim's notebook, document the measurement of protein C activation and human TM protein expression in rabbit vein grafts transduced with either the AdTmH5 or control adenoviral vectors. Figure 15, a page from Dr. Richard Sohn's notebook, demonstrates the construction of an adenoviral vector expressing I κ B, a potent NF- κ B inhibitor. Figures 16, a page from Dr. Richard Sohn's notebook, shows the measurement of NF- κ B activation in rabbit vein grafts.

9. Figure 17 documents the ability of the I κ B adenovirus in preventing TM down-regulation in response to inflammatory cytokines. Figure 18, a page from Dr. Rade's notebook, details the group of rabbit vein grafts that were transduced with the I κ B adenovirus. Figure 20 is

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a figure resulting from data demonstrating that over-expression of I κ B can reduce neointimal formation in rabbit vein grafts. Figure 21, a page from Dr. Sohn's notebook, shows that over-expression of I κ B also effectively inhibits NF- κ B activation in rabbit vein grafts.

10. We hereby further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. 1001), and that such willful false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Date: 10/30/06



Jeffrey J. Rade

Date: _____



Antony Kim

Date: 10/30/06



Richard Sohn

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Page 3

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Date: _____

Jeffrey J. Rade

Date: 2 Nov 2008


Antony Kim

Date: _____

Richard Sohn

Figure 1

Project No. 2

Book No. 1

TITLE *Cloning of human Thrombomodulin into AdLX*

On Page No. 1

Purpose: To make recombinant Adenovirus c human Thrombomodulin

1. Obtained plasmid clone of human Tm from ATCC (ATCC# 61348) F

ATCC 61348, 61349 - plasmid clone of human thrombomodulin <http://www.atcc.org/cgi-bin/STGate?lang=828%20%2fpub%2fexfiles%2fMB-CLONE1.TXT>Your query was:
thrombomodulin

Conf # S044066 F

\$105 + \$17.75 shipping

ATCC 61348, 61349 - plasmid clone of human thrombomodulin

ATCC 61348, 61349 - plasmid clone of human thrombomodulin, THBD

NAME: puc19TM15 (GDB:168893) [lambda HTM15]

DATABASE ACCESSION: DNA Seq. Acc. M16552

VECTOR: plasmid vector: pUC19

ORGANISM: THBD; Homo sapiens (human)

TISSUE: umbilical vein endothelial cells

GENE NAME: in insert THBD: thrombomodulin (GDB:119613)

CHROMOSOME: THBD 20: p11.2

DNA: THBD cDNA

CONSTRUCTION: Insert lengths (kb): THBD 3.70

Excise by: EcoRI or SalI

6.40

Sequence Position: DNA Seq. Acc.: M16552

MARKERS: ampR

DEPOSITORS: J-E Sadler

REFERENCES: Biochemistry 26: 4350-4357, 1987

J. Biol. Chem. 264: 20705-20713, 1989 (CIT:381458)

Genomics 5: 649-650, 1989 (CIT:13744)

DESCRIPTION: Restriction digests of the clone give the following sizes

(kb): EcoRI- 3.7 2.7; SacI- 6.5; Aval- 3.4 2.0, 0.2; XbaI- 5.9,

0.54 0.2 0.1 (ATCC staff)

There is 64% homology between this probe and bovine thrombomodulin. A

single band of 3.7 kb is detected in human placenta and endothelial

cell poly(A)+ RNA. The insert includes 146 nt of 5'-noncoding

sequence, an open reading frame of 1725 nt and 1779 nt of 3'-noncoding

sequence including 40 nt of Poly(A) tail. The insert contains the

following sites separated by (bp) (approx): EcoRI- 270- SmaI- 160-

PstI- 240- PstI- 210- PstI- 100- PstI- 530- KpnI- 310- PstI- 1080-

HindIII- 110- BamHI- 205- PstI- 475- EcoRI. (Biochemistry 26:

4350-4357, 1987)

GROWTH CONDITIONS: Medium 1227 37C

SHIPPED: 61348: freeze-dried Escherichia coli SURE

61349: dried purified DNA (200 ng)

PRICE CODE: 61348 D -- TIGR/ATCC SPECIAL COLLECTION OF HUMAN cDNA

CLONES

61349 D -- TIGR/ATCC SPECIAL COLLECTION OF HUMAN cDNA CLONES

To Page No.

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Figure 2

54

Project No.

Book No.

TITLE : TYPE OF RADAR AND ITS GRAPHS

From Page No. 10

Purpose: STUDY THE CONCEPT, SCHEMATIC DIAGRAM, PRACTICAL DESIGN
AND FORMULAS OF THE GYROSTABILIZED OPEN
LONGITUDINAL & PHOTOCOUPLED NIGHT CHARTS.

① RGAU-

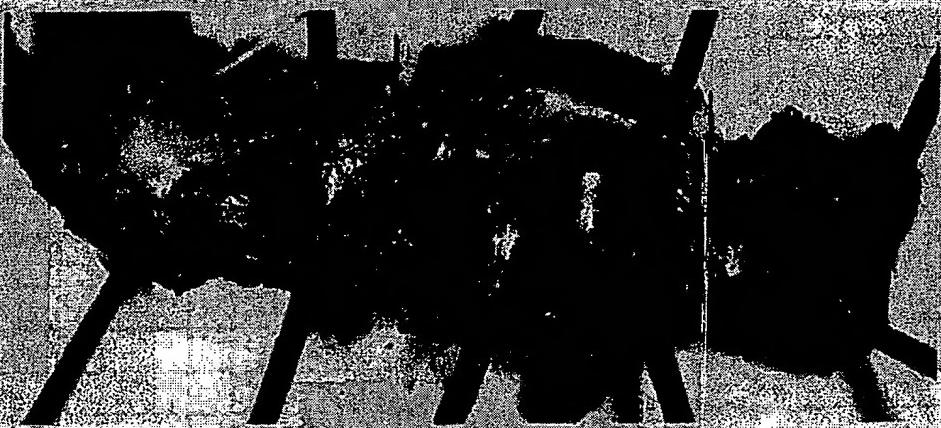
united

→ ununited

In = 20000 m-sec + Ad. CHARGE (500)

→ 40000 PPM (8x10¹⁰ PFU 1x0)

8x10¹⁰ PFU



united

BEST SOURCE = MOP CHANNEL

ARMED

② RGAU-4

→

Same ununited

united



Witnessed & Understood by me:

Date:

Invented by:

Date:

Recorded by:

Figure 3

Day 30
TM+1

1. T views in 1025
not in view at 1135

Ad-T-His

Clamp on 1143
Clamp off 1238

P. Genua and clamp 11
Collet size = no blow or leak 11

good longitudinal skin incision

Clamp 11 revealed huge vein in proximal graft

Repaired as above to save hemostasis
still patent

One finger width of skin was
dissolved

Sigel left O distal incision

> 77 well see

Please

See patient weekly - left open

HIP

75 X

P. No.
(
Reflux
Segment
Bgs
Gra
Please

Figure 4

Project No.		Book No.		TITLE	
From Page No.					
GKAR'S CUT TO API					
Stringer	Screws	Welded	Welded	Days	Notes
TMC-1				5	60
TMC-2				5	25
TMC-3				5	25
TMC-4				7	0
TMC-5				7	0
TMC-6				7	0
TMC-7				7	258
TMC-8				1	0
TMC-9				1	0
TMC-10				3	0
TMC-11				3	0
TMC-12A				10	0
TMC-12B				10	Sum
TMC-13				28	0
TMC-14				3	Sum
TMC-15				3	Sum
TMC-16				3	Sum
TMC-17				3	Sum
TMC-18				3	Sum
TMC-19				3	Sum
TMC-20				3	Sum
ANUAC-1				0	0
ANUAC-2				0	0
ANUAC-3				0	0
ANUAC-4				0	0
ANUAC-5				0	0
ANUAC-6				0	0
ANUAC-7				0	0
ANUAC-8				0	0
ANUAC-9				0	0
ANUAC-10				0	0
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ANUAC-280					

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GROUTS SENT TO APIB

SPEARIN

285 C-30, LT-13
 285 C-33, LT-14
 285 C-34, LT-15

SPEARIN
DONGHARVEST
DONG

Adcon-1 2853

Adcon-2 2855

Adcon-3 2860

Adcon-4 2861

Adcon-5 2862

Adcon-6 - 2863

Adcon-7 2863

TMTC-23 3861

TMTC-24 3865

TMTC-21 3862

TMTC-22 3861

Adcon-8 3205

Adcon-9 3206

TMTC-25 3863

CGW-2 3208

CGW-3 3209

CGW-5 3210

CGW-8 3211

CGW-10 3212

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[Signature]

Date

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Recorded by

Figure 6

DA 3

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Figure 7

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Project No. _____

Book No. _____

TITLE TM in RVG model

From Page No. _____

Digital Imaging of Thrombomodulin in RVG's !

Protocol :

Hypothesis #1: TM protein expression decreases ^{in RVG} over time when ^{grafted into} ^{arterial circulation}.

Reason :

Hypothesis #2 : TM protein production shifts the thrombore sistent balance of normal vasculature to cause a more thrombotic state.

Reason :

Hypothesis #3 : The prothrombotic state left by the reduction of TM is a factor in VG failure.

Reason :

Adobe

- ① shoot images via DMC
- ② adjust brightness in order to control for all vessels
- ③ mask the vessel \sim from endothelium

Sigma
Scan

- ④ measurement setting = # of pixels (area) calibration 333 to 1
- ⑤ measure # of pixels on vessel endothelium (choose orange for signal intensity threshold)
- ⑥ measure circumference of endothelium
- ⑦ record values including # pixels, circumference, + P/C

Quattro

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Date

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Date

To Page No.

Recorded by

GUARD SUT TO APIP

Project No.
Book No.

Figure 8

GUARD SUT

Specimen

Survey

HABITAT

DAY

28

VIRUS

Adenovirus

Dose

2.5 MO?

346

Adenov-10

Adenov-11

Adenov-12

Adenov-13

Adenov-14

Adenov-15

Adenov-16

Adenov-17

Clostridium

Clostridium N

Clostridium 157

Clostridium 16

Clostridium 19

bacillus

φ

3459

3460

3461

3462

3463

3464

3465

3466

3467

Start

Frozn sections, GRAFT, ANEST, control art. vici

1d

φ

φ

1°

bacillus

7d

φ

φ

2d

φ

φ

3d

φ

φ

1d

φ

φ

2d

φ

φ

3d

φ

φ

GUARD CLOTHS

control art. vici only
GUARD art. vici

GUARD only

To Page No.

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Invented by

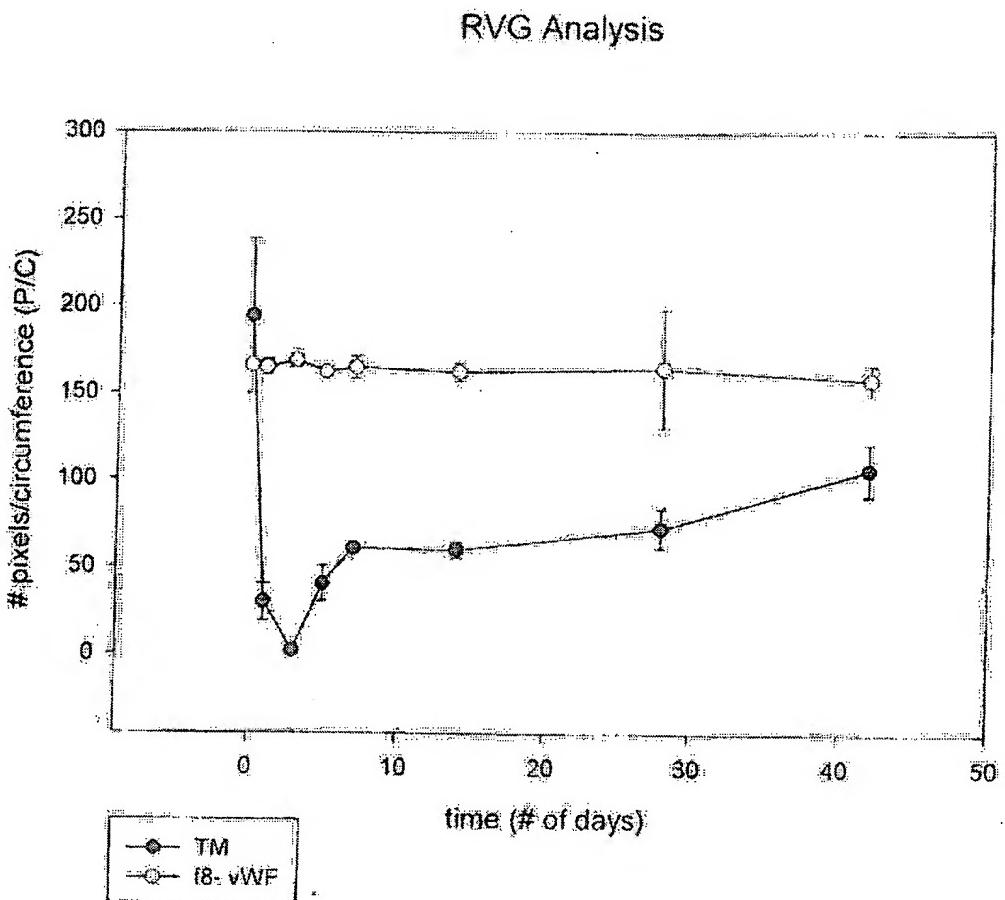
Date

Recorded by

TM & F8-VWF

Project No. _____
Book No. _____

Figure 9



Assessed & Understood by me,

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To Page No. _____

[Signature]

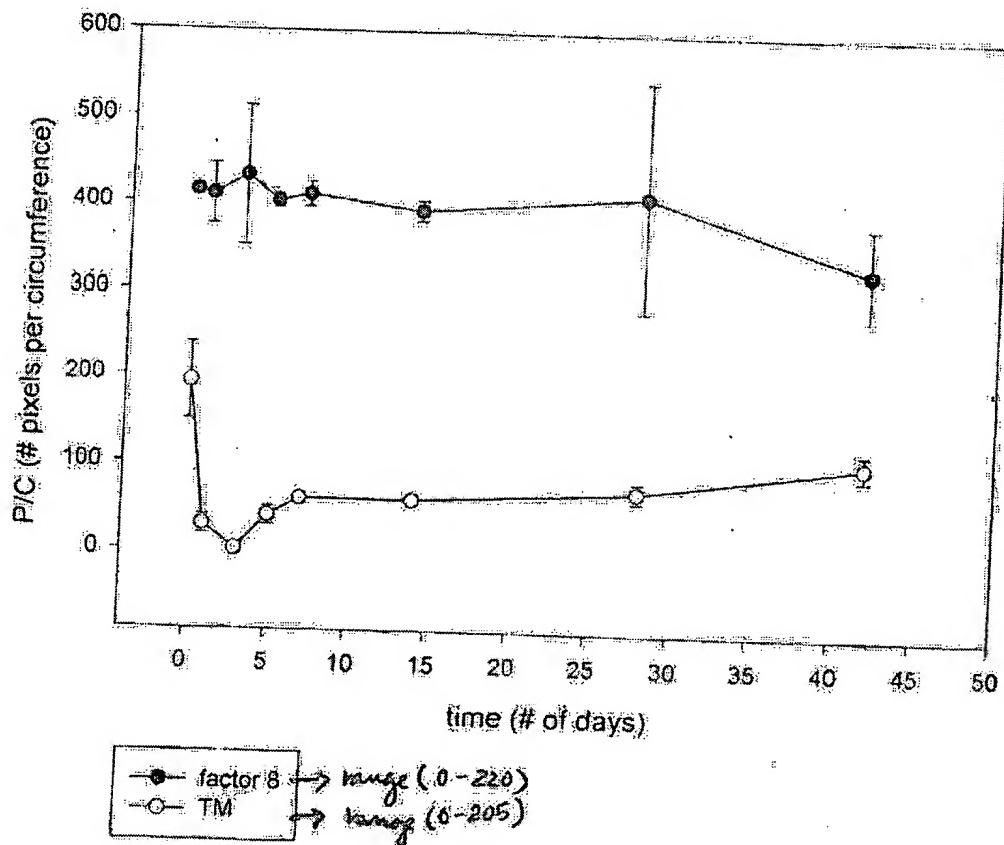
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Figure 10

Page No. _____

TM & Factor 8 stains

TM and F8



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To Page No.

Recorded by

Date

Figure 11

Project No.	Book No.	TITLE: GRANT, SUE TO APIS			
100.		Surf	Site	Vines	Waste
From Page No. _____					
Purfusen Fixus 10% Fom → 70% OTOH For Insecticide					
4017	Actimic #3				
4018	Actimic #4				
4019	Actimic #5				
4020	Actimic #6				
4021	Actimic #8				
Surf					
Purfusen Fixus 10% Fom → 70% OTOH For Insecticide					
4232	Actimic #12				
4233	Actimic #15				
4234	Actimic #16				
Hand Camera to MPV					
4508	C-89				
4509	C-90				
4510	Acticon #33				
4511	Acticon #36				
Purfusen Fixus 10% Fom → 70% OTOH Surf					
4512	Actimic #14				
4513	Acticon #21				
4514	C-74				
4515	C-81				
Purfusen Fixus 10% Fom → 70% OTOH Surf					
4517	Acticon 35				
4518	Acticon 41				
4519	C-91				
4520	C-92				
4521	C-93				
Acticon Purfusen Fixus 10% Fom → 70% OTOH Surf					
Witnessed & Understood by me _____ Date _____					
To Page No. _____					

Figure 12

186

Project No. _____

Book No. _____

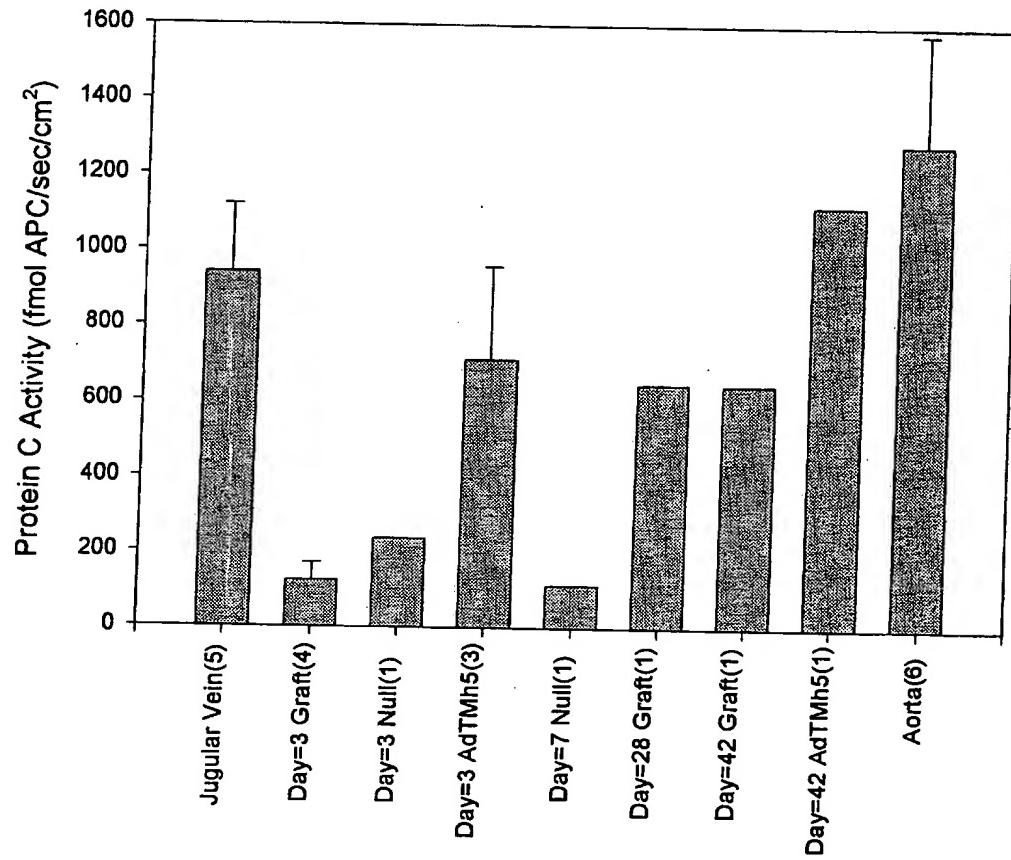
TITLE

PC Assay + Th Act. Assay

From Page No.

Thurs.

Protein C Assay (graph)



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Date

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Date

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Fri

① PC Activation Assay :

Figure 13

PC Activation Assay AdTMh5 #25 (day 7)

[thrombin] nM 10

[PC] uM 1

RVG (7 day) blank

AdTMh5#25	522	16.29
AdTMh5#25 - blank	505.71	

avg.	505.71	16.29
aPC	0.468275065	
aPC(fmoles/min/cm ²)	2498.2474717	

aPC Standard Curve

uM aPC mOD/min

Regression Output:

0	0.857	Constant	0.001813143
0.05	55.4	Std Err of Y Est	0.006520627
0.1	107.4	R Squared	0.998984198
0.2	213.8	No. of Observations	7
0.3	311.6	Degrees of Freedom	5
0.4	428.4		
0.5	549.2	X Coefficient(s)	0.00092239
		Std Err of Coef.	1.31539E-05

To Page No.

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Now TM ELISA for AdTMh5 + AdNull (American diagnostics inc kit #83)

Figure 14

THROMBOMODULIN ELISA

	<u>sample</u>	<u>OD (A450)</u>	<u>Dilution factor</u>	<u>TM</u>	<u>corrected TM</u>	<u>TM/BSA</u>
<u>AdTMh5</u>						
<u>day 3</u>						
	TM9	1.21	20	3.20804	24.20000	39.18483
	TM11	1.23	50	3.27142	163.57088	185.85785
	TM23	1.359	50	3.68020	184.00989	108.11640
<u>day 7</u>						
	TM25	3.166	50	9.40629	470.31439	354.42972
	TM27	2.466	50	7.18810	359.40506	149.04811
<u>day 14</u>						
	TM19	0.681	10	1.53173	15.31726	9.36070
	TM20	0.781	10	1.84861	18.48609	7.40715
	TM21	0.791	10	1.88030	18.80298	9.96799
<u>day 28</u>						
	TM22	2.187	50	6.30400	315.19977	231.53710
	TM29	1.162	50	3.05594	152.79683	66.64825
	TM30	1.148	50	3.01157	150.57865	65.59129
<u>day 42</u>						
	TM18	0.437	10	0.75853	7.58529	11.80432
<u>AdNull</u>						
<u>day 3</u>						
	NULL23	0.187	2	0	0	0
	NULL28	0.16	2	0	0	0
	NULL43	0.204	2	0	0	0
<u>day 7</u>						
	NULL25	0.195	2	0	0	0
	NULL27	0.206	2	0	0	0
	NULL31	0.201	2	0	0	0
<u>day 14</u>						
	NULL34	0.258	2	0	0	0
	NULL37	0.193	2	0	0	0
	NULL40	0.149	2	0	0	0
<u>day 28</u>						
	NULL29	0.172	2	0	0	0
	NULL36	0.194	2	0	0	0
	NULL39	0.165	2	0	0	0
<u>day 42</u>						
	NULL19	0.181	2	0	0	0
	NULL22	0.197	2	0	0	0
	NULL24	0.188	2	0	0	0

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Figure 15

From Page No. _____

Primer "RSV1":

AGCACC GTGCA TGCC GATT GAT TGA

T5 insert then fragments \rightarrow ~1300 - 1400 bp.

PCE

(1) (2)

RSV

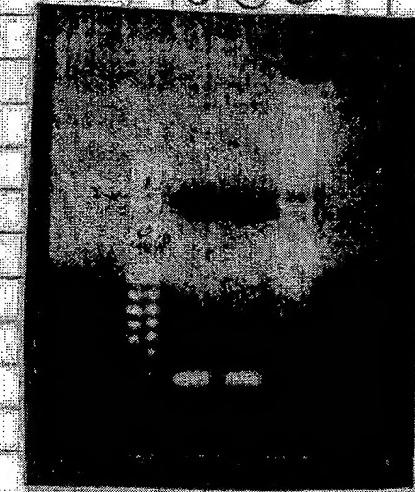
pAdex R 1KB wt

WT

(3)

Viral plate is overlayed
2nd layer MEM agarose.

(1) (2) (3)



→ No insert in viruses
→ Ctrl (shuttle plasmid)
verifying PCE works

→ To send out plates is for CMV, ΔS 1KB

① Pick up 6 plaques and innoculate 6 well-plate of 293 cells

② Thaw CEF cells

③ Digest [pAdex R 1KB wt] or [pAdex R 1KB ΔS] in lysis this recombinant is 45% will be more efficient

Witnessed & Understood by me:

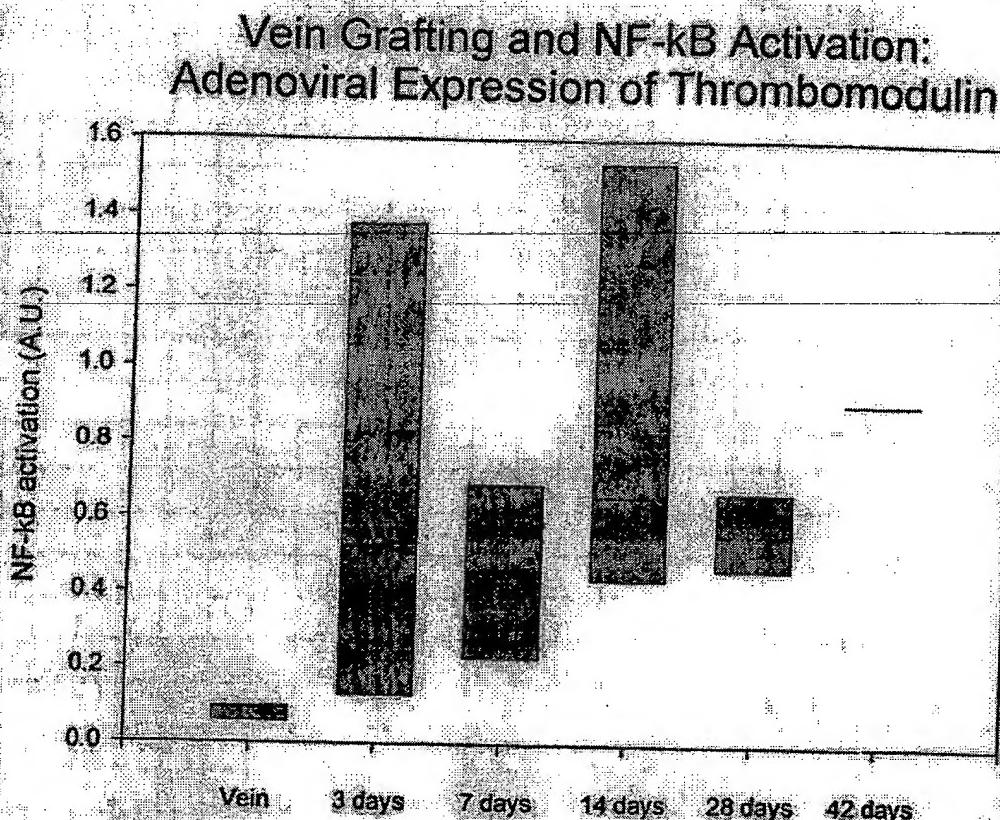
Date

Invented by

Date

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To Page No. _____



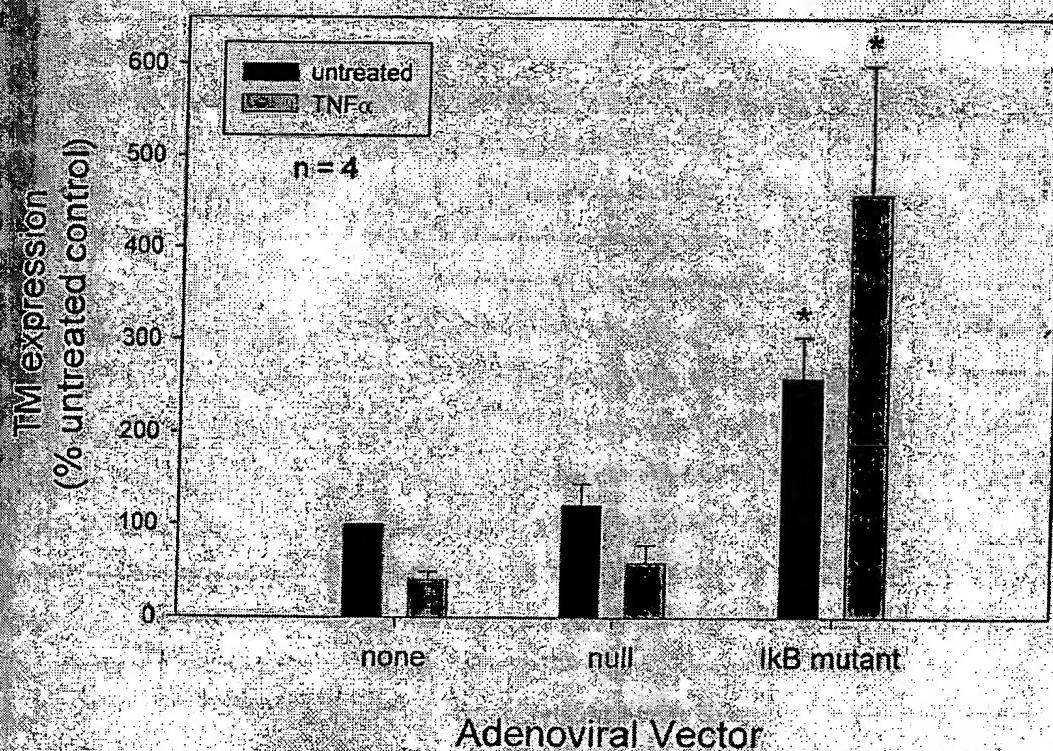
Plated 397 cells
 50% NUNCL 50 plates, 60mm
 T-plate 150mm
 ready confluent \rightarrow infect 2 virus
 AdTM
 90 min
 Cuvette
 dilution 2.5 ml
 9%
 CVL
 100 Moi (5 ul)
 in 600 ul

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Figure 17

	ctrl	TNF	null	null + TNF	IkBm	IkB + TNF
gel 1	598	274	519	148	1262	1290
gel 2	249	63	430	228	331	1826
gel 3	286	84	423	265	950	1918
gel 4	1088	668	841	288	1754	2330
as % ctrl	100	45.8194	86.7893	24.74916	211.0368	215.7191
	100	25.3012	172.6908	91.56627	333.7349	733.3333
	100	29.37063	147.9021	92.65734	332.1678	670.6294
	100	61.39706	77.29779	26.47059	161.2132	214.1544
mean	100	40.47207	121.17	58.86084	259.5382	458.459
S.D.		0.16.53125	46.46073	38.40392	87.17875	282.3589
S.E.M.		0.8.265623	23.23036	19.20196	43.58938	141.1794
t-test						
vs. control			0.397268		0.010579	
vs. null					0.031108	
vs. null-TNF						0.030975

Inhibiting NF- κ B Activation In HUVEC Upregulates TM and Prevents TM Downregulation By TNF α



* P < 0.05, compared to respective null-transduced

Recorded by

Figure 18

Project No.		TITLE GRAPHS SNT DO APR																																																	
03	Book No.																																																		
From Page No. 103																																																			
<p>$10\% \text{ Form Fixer} \rightarrow 20\% \text{ Urine}$</p> <table border="1"> <thead> <tr> <th>Rabbit</th> <th>Serum</th> <th>Hair</th> <th>Virus</th> <th>Dose</th> <th>Time</th> </tr> </thead> <tbody> <tr> <td>1KB-2</td> <td>6234</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-3</td> <td>6235</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-9</td> <td>6236</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-10</td> <td>6237</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-4</td> <td>6238</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-12</td> <td>6239</td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td>1KB-13</td> <td>6230</td> <td></td> <td></td> <td></td> <td></td> </tr> </tbody> </table> <p><u>Fraction</u></p> <p><u>Serum</u></p> <p><u>Hair</u></p> <p><u>Growth</u></p> <p><u>AyR 103</u></p> <p><u>SNT APR 10</u></p> <p>To Page No.</p>				Rabbit	Serum	Hair	Virus	Dose	Time	1KB-2	6234					1KB-3	6235					1KB-9	6236					1KB-10	6237					1KB-4	6238					1KB-12	6239					1KB-13	6230				
Rabbit	Serum	Hair	Virus	Dose	Time																																														
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Figure 19

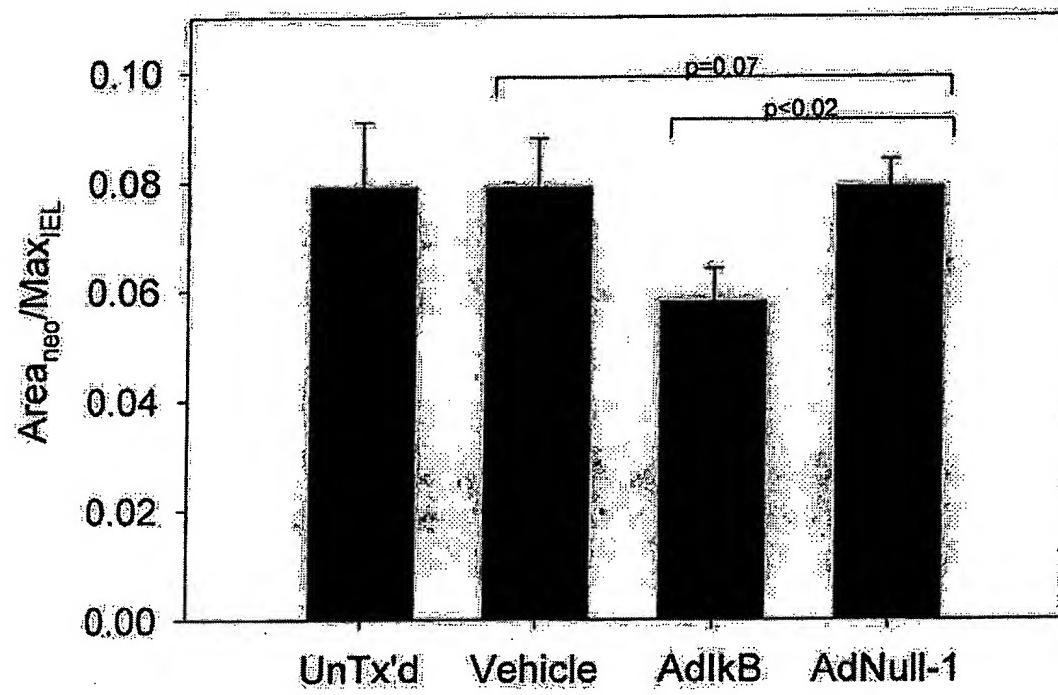
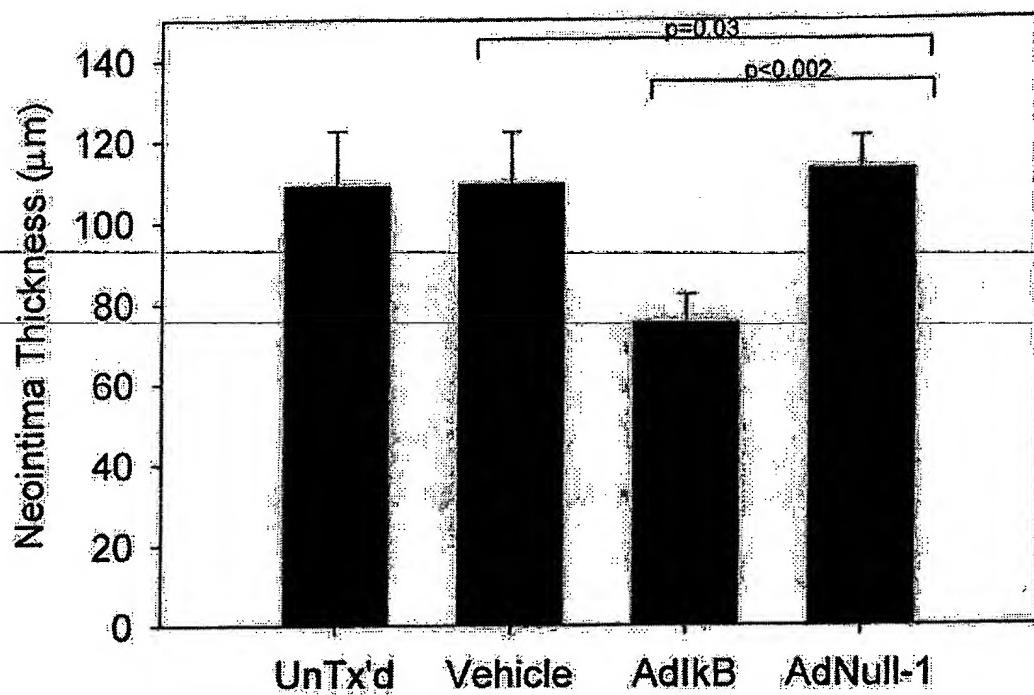
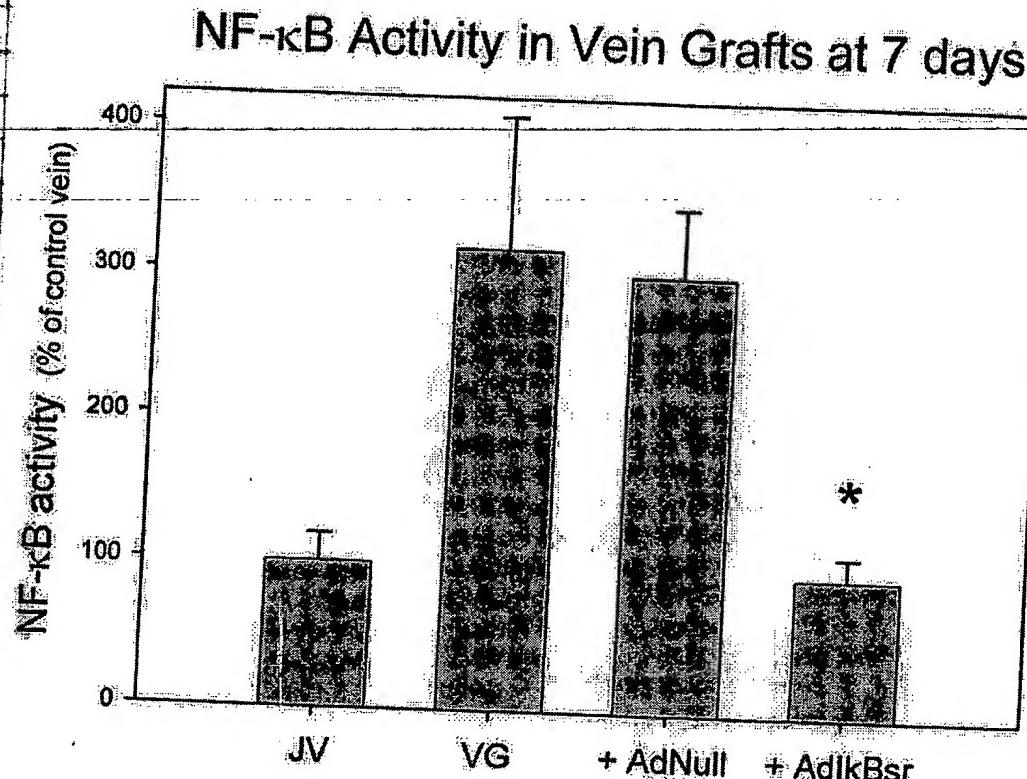


Figure 20



* P=0.014, as compared to AdNull-transduced vein graft.

Reagents & Transducing NUVEG = 7 following kits:

① Tfx PEI-RGD (Qbiogene Molecular Biology)

② TfFx - 50 (Promega)

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